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Evidence for Apoptosis in Advanced Human Atheroma

Colocalization with Interleukin-1β-Converting Enzyme

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This study sought evidence for apoptosis, a form of programmed cell death, in human atheromatous coronary and carotid arteries. Markers for apoptotic cells included* in situ *terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), genomic DNA electrophoresis, and morphological analysis. Intimal lesions contained more TUNEL$^+$ cells (34 ± 6%, n = 8) than non-atherosclerotic arterial intima (8 ± 3%, n = 5, P < 0.05). The tunica media of the diseased arteries had a percentage of TUNEL$^+$ cells (5 ± 1%) similar to that in the normal vessels (3 ± 1%, N.S.). Oligonucleosomal DNA fragments were visualized in extracts from 12 atheromatous plaques but in none of 5 non-atherosclerotic vessels. Both smooth muscle cells (SMC) and macrophages, two major cell types in the atherosclerotic intima, bore markers of apoptosis, but with different patterns, as determined by double histochemical labeling for cell types and TUNEL. The TUNEL$^+$ SMC localized mainly in the fibrotic portion of the atheroma, whereas TUNEL$^+$ macrophages clustered near or within the lipid-rich core of the lesion. Atheromatous lesions expressed mRNA encoding interleukin-1β-converting enzyme (ICE), a mammalian cell death gene, as demonstrated by reverse transcriptase polymerase chain reaction. Immunohistochemistry revealed that ICE localized in regions of TUNEL$^+$ SMC and macrophages. TUNEL$^-$ cells showed little or no immunoreactive ICE. These data point to a role for apoptosis in regulation of cell accumulation during atherogenesis and suggest involvement of ICE in SMC death in fibrous regions of complex atheroma, and in macrophage death in the lipid-rich core of the lesion. Apoptosis of vascular cells in fibrous cap may impede maintenance or repair of the matrix in this region and affect stability of the plaques. (Am J Pathol 1995, 147:251–266)

Focal thickening of the arterial intima caused mainly by accumulation of cells, lipids, and connective tissue characterizes the pathogenesis of atherosclerosis.[1] Traditional thought about the mechanism for intimal thickening accords an important role to proliferation of smooth muscle cells (SMC).[2] We have learned much over the last decades about growth factors and cytokines that promote SMC proliferation.[1,3] Yet, advanced atherosclerotic lesions such as atheroma contain few proliferating cells.[4] Analysis of proliferation markers reveals a low rate of SMC proliferation, similar to that of macrophages, another major cell type in the complex atheromatous lesions.[5,6] In addition to growth stimulators, cells in the vessel wall may produce inhibitors of SMC proliferation such as prostanoids,[7] interferon-γ (IFN-γ,)[8] and nitric oxide.[9,10] Thus, although SMC proliferation likely contributes to development of atherosclerotic lesions, particularly during earlier phases, the role of cell proliferation in established plaques remains uncertain.

On the other hand, cell death characterizes the advanced atherosclerotic lesions, as clearly stated by Virchow[11] in the mid-19th century. The center of atheroma contains, in addition to lipids, dead cells or cell debris, and therefore bears the name "necrotic center" or "lipid core". Fibrosis also occurs as atherosclerosis progresses, often yielding a lesion containing a dense extracellular matrix with a relatively low density of cells in advanced plaques. Cell death may

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provide a means for conversion of a hypercellular lesion to the more cytopenic fibrotic atheroma.

Apoptosis, a form of programmed cell death involved in tissue morphogenesis and homeostasis,[12,13] may constitute an alternative mechanism for cytokine-mediated cell death in atherosclerotic lesions[3] and for focal fibromuscular dysplasia of small coronary arteries.[14] Recent studies support this notion by documenting induction of apoptosis of vascular cells by deregulated expression of the oncogene c-*myc*[15], inhibition of protein kinase C,[16] deprivation of growth factors,[17] or stimulation with proinflammatory cytokines.[18] Apoptosis also serves as a major factor for determination of SMC number in the arteries of the neonatal lamb.[19]

In contrast to necrosis (non-programmed, sudden cell death), initiation of apoptosis frequently requires *de novo* gene expression and new protein synthesis.[20] Among the death-regulating genes, a mammalian cysteine protease catalyzing conversion of precursor of the proinflammatory cytokine, interleukin-1β (IL-1β), into a bioactive form,[21,22] holds considerable interest because of the potential role of this or related enzymes in induction of apoptosis.[23,24] Overexpression of this IL-1β-converting enzyme (ICE) gene by cDNA transfection causes apoptosis in the rodent fibroblast cell line, Rat-1.[24] The functions of ICE and its cDNA sequence resemble that of *ced*-3, a gene involved in cell death in the nematode *Caenorhabditis elegans*.[23,24] Vascular endothelial cells and SMC can produce IL-1β,[3] and experimental atherosclerotic lesions contain both isoforms of IL-1 mRNA.[25] However, it is unknown whether vascular or other cells express ICE in human atherosclerosis.

To seek evidence for apoptosis and explore the role of the mammalian death gene ICE in vascular apoptosis in atheroma, we have investigated apoptosis and ICE expression in both normal and atherosclerotic human arteries. We found evidence for increased apoptosis of SMC and macrophages in different regions of human atheroma. The mammalian cell death gene, ICE, colocalized with the cells bearing markers of apoptosis. These data shed light on the role of apoptosis in regulation of vascular cell accumulation, and suggest involvement of the ICE-IL-1β pathway in apoptotic death of SMC and macrophages in human atheroma.

Materials and Methods

Arterial Specimens

Atherosclerotic plaques of human coronary and carotid arteries with atherosclerosis were obtained from patients with severe ischemic coronary heart disease and receiving heart transplantation or from patients undergoing carotid endarterectomy at the Brigham and Women's Hospital, Boston. The arterial specimens were immersed in ice-cold Hanks' solution immediately after removal, washed, and fixed in 10% formalin for paraffin section or snap-frozen in optimal cutting temperature tissue processing medium (O.C.T., Miles Diagnostics, Elkhart, IN) with liquid nitrogen and stored in −80°C for cryostat sectioning. In some experiments, a portion of carotid plaques was used for isolation of genomic DNA and of RNA. Normal aortas were obtained from the Pathobiological Determinants of Atherosclerosis in Youth archive or from transplant donors and the control coronary arteries from individuals receiving heart transplants for idiopathic dilated cardiomyopathy without coronary arterial disease. The study of normally discarded human tissues was approved by the Institutional Human Investigation Review Committee.

Cell Isolation and Culture

Human vascular SMC were isolated and cultured as previously described.[26,27] Briefly, SMC were grown in Dulbecco's minimum essential medium supplemented with 10% fetal calf serum and antibiotics. At confluence, cells were replated by trypsinization and passage 2 to 4 cells were used for experiments. Human mononuclear cells were isolated from buffy coats or leukocyte-rich fractions collected from peripheral blood of health donors by leukapheresis, followed by Ficoll Hypaque (Pharmacia LKB Biotechnology, Piscataway, NJ) centrifugation.[28] Monocytes were then separated from lymphocytes by adherence to culture flasks and induced to differentiate into macrophages by incubation in RPMI 1640 medium containing 10% fetal calf serum for 2 weeks. To induce apoptosis, cells were treated simultaneously with the recombinant cytokines, IL-1β, tumor necrosis factor-α (TNF-α), and IFN-γ (Genzyme, Cambridge, MA).

In Situ *Detection of Apoptotic Cells*

Paraffin sections of arterial tissues were deparaffinized by immersing the slides in xylene twice for 5 minutes and then rehydrated in 100, 95, 75, and 0% ethanol for 3 minutes of each. After rehydration, sections were washed in phosphate-buffered saline (PBS) containing 0.5% H_2O_2 to inactivate endogenous peroxidase, and then incubated with 20 μg/ml of proteinase K in PBS. DNA fragments in the tissue sections were determined using an ApoTag

in situ apoptosis detection kit (Oncor, Inc., Gaithersburg, MD). The labeling procedure was performed following the supplier's instructions with minor modifications. In principle, the enzyme, terminal deoxynucleotidyl transferase (TdT), which catalyzes a template-independent addition of deoxyribonucleotide to 3′-OH ends of DNA, was used to incorporate digoxigenin-conjugated dUTP to the ends of DNA fragments. The signal of TdT-mediated dUTP nick end labeling (TUNEL) was then detected by an anti-digoxigenin antibody conjugated with peroxidase, a reporter enzyme that catalytically generates a brown-colored product from the chromogenic substrate diaminobenzidine. The labeling conditions were optimized by adjusting incubation time and concentrations of TdT. After TUNEL, counterstaining was performed by immersing the slides in 0.5% methyl green in 0.1 mol/L sodium acetate solution (pH 4.0) for 5 minutes at room temperature. The slides were washed, dried, and mounted in Permount medium. Cell counting was performed under a light microscope. For each arterial specimen, at least four sections were examined and 400 cells were counted in random fields of each section at high power. The cells with clear nuclear labeling were defined as TUNEL-positive ($TUNEL^+$) cells. The apoptotic index was calculated as percentage of $TUNEL^+$ cells using the following formula. Apoptotic index = 100 × (number of $TUNEL^+$ cell nuclei/number of total cell nuclei).

Immunohistochemistry

After fixation in acetone for 10 minutes at −20°C, frozen sections were incubated with 1:50 normal horse serum for 1 hour at room temperature, washed once in PBS, and then stained with mouse monoclonal antibodies against human muscle-α-actin (HHF35, DAKO, Carpinteria, CA) and the macrophage marker CD68 (DAKO-CD68, KP1, DAKO). Both monoclonal antibodies were used at a dilution of 1:200. A polyclonal rabbit antibody recognizing human ICE (provided by Dr. D. K. Miller, Merck Research Laboratories, Rahway, NJ) was used at a dilution of 1:200. After being washed three times in PBS, the slides were incubated with biotin-conjugated secondary antibodies; biotinylated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA) was used for detecting the stains with antibodies against actin and CD68, and biotinylated goat anti-rabbit IgG for the stain of anti-ICE. Immunostains were visualized by using an avidin-alkaline phosphatase-substrate system (Vectastain ABC Kit, Vector Laboratories). Irrelevant mouse IgG and normal rabbit serum were used for control experiments. To identify cell types undergoing apoptosis, double staining was performed by combining TUNEL and immunohistochemistry with antibodies against actin, CD68, or ICE. Paraffin sections were used for the double staining, because apoptotic cells were poorly detected by the TUNEL method in frozen sections. After deparaffinization and rehydration, the slides were stained for apoptotic cells by TUNEL and then immunostained (see above).

DNA Isolation and Electrophoresis

Genomic DNA was isolated from arterial tissues (200 mg) or cultured cells (10^7 cells) in 2 ml DNA extraction solution containing 20 mmol/L Tris-HCl pH 7.4, 0.1 mol/L NaCl, 5 mmol/L EDTA, and 0.5% sodium dodecyl sulfate (SDS). The lysate was then incubated with 100 μg/ml of freshly prepared proteinase K (Sigma Chemical Co., St. Louis, MO) at 56°C for 16 hours. After incubation, cell lysates were centrifuged at 2000 × *g* for 5 minutes. Supernatants were collected and mixed well with equal volume of phenol/chloroform (1:1). After centrifugation at 20,000 × *g* for 10 minutes, DNA in the upper aqueous phase was incubated with 5 μg/ml of DNAse-free RNase A (Sigma Chemical Co.) at 37°C for 30 minutes, to eliminate RNA that might potentially contaminate the DNA preparation. DNA was reextracted with phenol/chloroform and precipitated at −20°C overnight in isopropanol followed by centrifugation at 10,000 × *g* at 4°C for 20 minutes. The resulting DNA pellet was washed with 75% ethanol and dissolved in 10 mmol/L Tris-HCl and 1 mmol/L EDTA and concentration was determined at 260 nm by spectrophotometry. Five μg DNA was loaded into 1.5% agarose gel containing 1 μg/ml ethidium bromide. DNA electrophoresis was carried out at 80 V for 1 to 2 hours, and bands were visualized under ultraviolet (UV) light.

RNA Isolation

Total RNA was isolated from arterial tissue and cultured cells using the method of Chomczynski and Sacchi,[29] with modification. Briefly, tissue or cells were lysed in an extraction solution containing 4 mol/L guanidinium isothiocyanante, 100 μmol/L 2-mercaptoethanol, 25 mmol/L sodium citrate (pH 7.0), and 0.5% SDS, followed by addition of 0.1 volume sodium acetate (2 mol/L, pH 4.0) and extraction

with phenol (1 volume) and chloroform-isoamyl alcohol 49:1 (0.2 volume). RNA at the upper phase was collected and precipitated in ethanol. After centrifugation for 20 minutes at 20,000 × *g*, RNA pellet was dissolved in diethylpyrocarbonate-treated water, and concentration was quantitated at 260 nm. The integrity of RNA was determined by electrophoresis.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed as previously described.[10] Briefly, RNA (200 ng) was reverse transcribed into cDNA by incubating with 2.5 U/ml Moloney murine leukemia virus reverse transcriptase (Perkin Elmer, Norwalk, CT) at 42°C for 30 minutes in 20 μl RT buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3) containing 2.5 mmol/L random hexamers, 1 mmol/L of each dNTP, 1 U/ml RNAsin. After denaturation at 95°C for 2 minutes, cDNA was subjected to PCR amplification. The PCR reaction was composed of 5′ and 3′ primers (20 pmol for each), 200 μmol/L of each dNTP and 2.5 U Taq DNA polymerase (AmpliTaq, Perkin Elmer-Cetus, Norwalk, CT) in 100 μL of RT buffer with 2 mmol/L $MgCl_2$. PCR amplification for 30 cycles was performed on a Perkin Elmer-Cetus DNA thermal cycler. Each PCR cycle consisted of 94°C for 1 minutes, 55°C for 30 seconds, and 72°C for 2 minutes. A 10-minute elongation step at 72°C was added to the last cycle. PCR products were analyzed by agarose electrophoresis in 2% gel and visualized by ethidium bromide staining. Table 1 shows the sequences of the PCR primers for ICE[30] and keratinocyte transglutaminase (TG).[31]

Statistical Analysis

Statistical difference between means was evaluated using Student's *t* test. *P* values less than 0.05 were considered significant.

Results

Few Cells in Normal Coronary Arteries Bear Markers of Apoptosis

Apoptotic cells contain fragments of genomic DNA in their nuclei. The TUNEL method can detect these cells *in situ* by labeling the ends of DNA fragments.[32,33] We employed this method to analyze apoptosis in human arterial tissues by incorporating digoxigenin-conjugated dUTP into the DNA fragments with the enzyme TdT. A representative micrograph of a morphologically normal coronary artery shows few cells with an intense TUNEL stain (Figure 1, brown color). In the TUNEL$^+$ cells, the stain localized in cell nuclei rather than in their cytoplasm (Figure 1c). Counterstaining with methyl green visualized the cells whose nuclei were not stained with TUNEL (Figure 1, b and c). Omission of the enzyme TdT abrogated TUNEL staining (Figure 1d). In normal arterial tissue, the majority (>70%) of TUNEL$^+$ cells appeared in the intima (Figure 1, Table 2) where they gathered subendothelially (Figure 1, b and c). In contrast, TUNEL stained few cells in the tunica media (Figure 1b, Table 2). The overall level of TUNEL$^+$ cells was <4%, suggesting that apoptosis, a physiological process for adult tissue turnover, occurs at a limited rate in the normal arteries.

Many Cells in the Intima of Atheromatous Arteries Bear Markers of Apoptosis

Figure 2 shows the result of TUNEL staining in a coronary artery with an eccentric atherosclerotic lesion containing marked thickening of the intima and an adjacent region with a relatively normal morphology. The less involved, normal-appearing portion of the artery contained few TUNEL$^+$ cells (Figure 2, a and b). However, the number of TUNEL$^+$ cells increased in the intimal region with greater thickening (Figure 2a). In contrast to the intima, the tunica media of both the normal and diseased arteries contained few TUNEL$^+$ cells (Figure 2c, Table 2).

Table 1. *Sequences of Oligonucleotides Used as Primers for RT-PCR*

Templates	Primers	Sequences	Products
ICE	Sense	GGAAATTACCTTAATATGCAAGAC	399 bp
	Antisense	CATGAACACCAGGAACGTGCTGTC	
TG	Sense	TATGGCCAGTGCTGGGTCTTTGCT	388 bp
	Antisense	CACCTTGTCACTATTCACCTCAGC	

Oligonucleotide sequences are presented from 5′ to 3′.

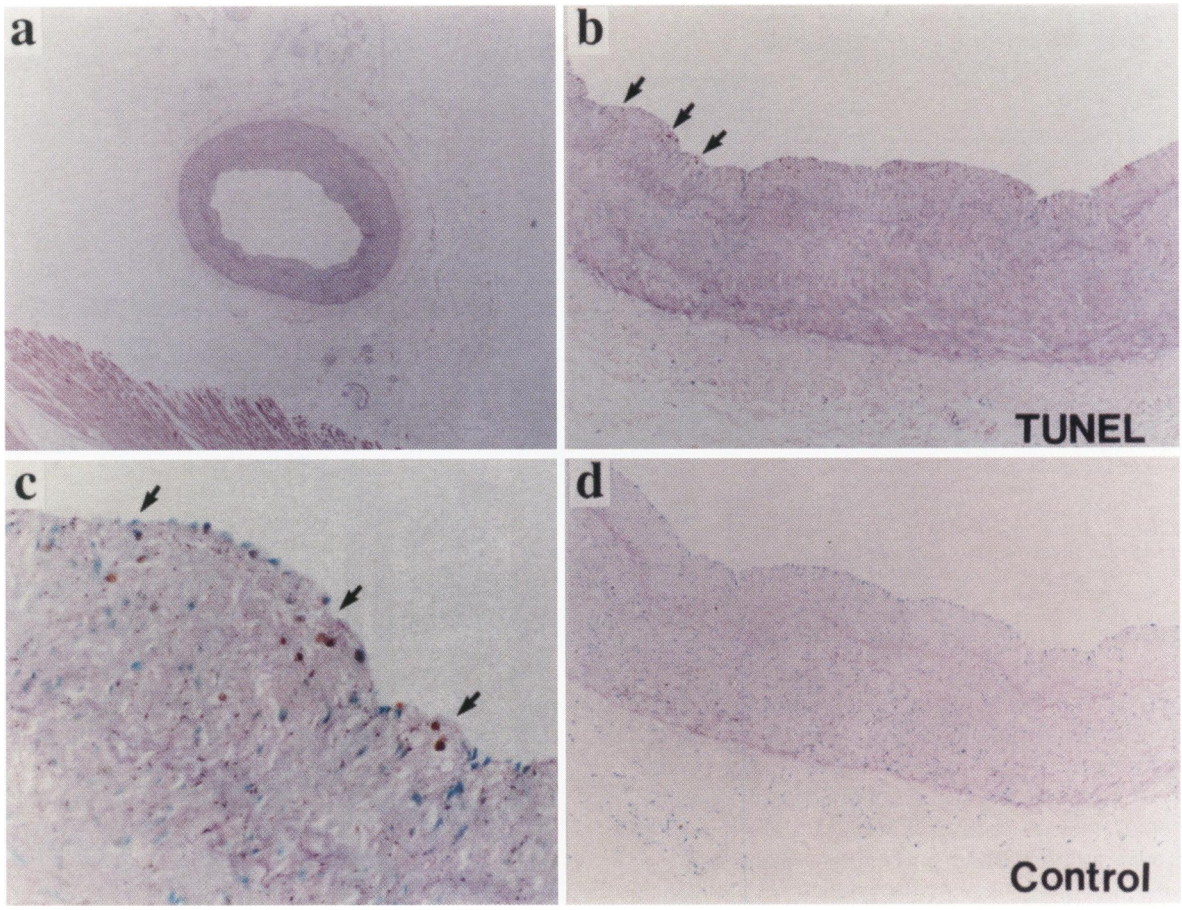

Figure 1. *TUNEL of normal human coronary artery. Cross sections of a human coronary artery were stained by TUNEL with digoxigenin-conjugated dUTP and the enzyme TdT* (**a–c**). *TUNEL⁺ cells were visualized with a peroxidase-substrate system showing brown nuclei. Methyl green counterstaining revealed green-blue nuclei.* **c** *shows a high power view of the field indicated by arrows in* **b**. *Omission of TdT abrogated the TUNEL staining* (**d**). *Note the subendothelial localization of TUNEL⁺ cells in the arterial intima* (**b** *and* **c**, **arrows**). *Original magnifications:* **a**, *×20;* **b** *and* **c**, *×100;* **d**, *×400.*

Table 2. *Vascular Cells Bearing Markers of Apoptosis in the Intima and Media of Human Coronary Arteries with or without Atheroma*

	Apoptotic index (%)		
Localization	Normal (n = 5)	Atheroma (n = 8)	*P* value*
Intima	8 ± 1	34 ± 5	<0.05
Media	3 ± 1	5 ± 1	N.S.

Cross sections of human arteries with or without atheroma were stained by TUNEL. Counterstaining was carried out with methyl green. At least four sections of each arterial segment were examined under a light microscope. 400 cells were counted in random fields. Apoptotic index was calculated by dividing number of TUNEL⁺ cells by total cell number. Data represent means ± SD.

*Comparison of means between the normal and atheromatous arteries. N.S., not significant.

A similar situation pertained to carotid atheromatous plaques that contain a lipid-rich core, a fibrous cap covering the lipid core, and a shoulder connecting to normal tissue of the artery. Numerous TUNEL⁺ cells existed in intimal lesions of the arteries, particularly near or within the lipid-rich core (Figure 3), where ~50% of cells appeared TUNEL⁺ (Table 3). Interestingly, we observed intense TUNEL stain in the extracellular compartment of the lipid core (Figure 3), suggesting accumulation of DNA fragments released from the dead cells in this region. Surprisingly, the fibrous cap also contained many TUNEL⁺ cells (Figure 3b). The presence of TUNEL⁺ cells in the thin fibrous cap implies that the cell death occurs in this region of structural weakening where plaques commonly rupture. TUNEL⁺ cells also localized subendothelially in the shoulder of the plaque (Figure 3). By contrast, the adjacent uninvolved tissue contained few TUNEL⁺ cells (Figure 3, b, d, and e).

To confirm the selectivity of the TUNEL stain used here, we analyzed the sections of normal tonsil tissue using the TUNEL method under the same conditions

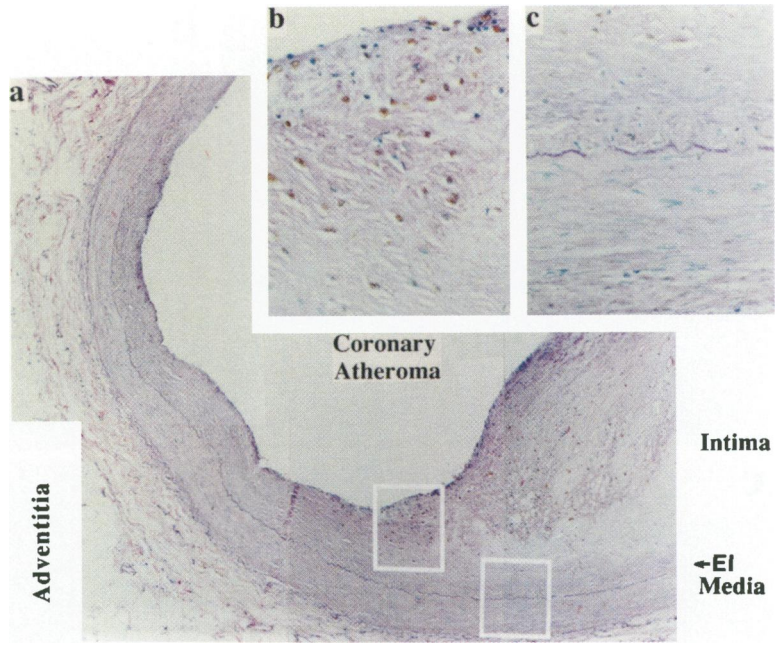

Figure 2. *TUNEL of atheromatous human coronary artery. Cross sections of a human atheromatous artery were stained by TUNEL with a peroxidase-substrate detection system yielding brown nuclei. Counterstaining by methyl green shows green-blue nuclei. Note the numerous TUNEL⁺ cells in the lesional intima* (b) *but not in the media* (c). (b *and* c) *High power micrographs of the intimal* (left) *and medial* (right) *regions indicated by respective-boxes in* a. *EI, elastica interna. Original magnifications:* a, *×100;* b *and* c, *×1000.*

applied to atherosclerotic lesions. The germinal center of tonsil lymphoid tissue, which usually exhibits high levels of apoptosis, contained many TUNEL$^+$ cells, whereas the tissue surrounding the germinal center showed no or few TUNEL$^+$ cells (Figure 4). Similar to the TUNEL$^+$ cells in the atheromatous artery, the TUNEL$^+$ tonsil cells exhibited morphological features typical of apoptosis, such as condensation of cytoplasm and chromatin (pyknosis)[34] and nuclear breakdown into discrete fragments (karyorhexis)[34] (Figure 4). TUNEL selectively stained these cells with the apoptotic nuclei but not the cells with a normal morphology (Figure 4).

Genomic DNA Breaks Down into Internucleosomal Fragments in Atheroma

The production of mono- or oligonucleosomal DNA fragments at multiples of 180 to 200 bp resulting from cleavage of genomic DNA by an endonuclease(s) furnishes a biochemical criterion distinguishing apoptosis from necrosis. To verify the occurrence of apoptotic cell death in the atheromatous lesions, we analyzed genomic DNA isolated from carotid atheromatous plaques of 12 patients (Figure 5). Although the degree of DNA fragmentation varied from one individual to another, each DNA sample contained oligonucleosomal DNA fragments (Figure 5). By contrast, genomic DNA isolated from the normal arterial tissue showed little fragmentation (Figure 5b). The pattern of DNA fragmentation in the atheromatous tissue resembled that in the vascular SMC treated with a combination of the cytokines, 100 U/ml IL-1β, 400 U/ml TNF-α, and 400 U/ml IFN-γ, which induced the production of internucleosomal DNA fragments (Figure 5a).

Both Macrophages and SMC Bear Markers of Apoptosis in Atheroma

To identify cell types that undergo apoptosis in atheroma, we performed double staining by a combination of TUNEL and immunohistochemistry with monoclonal antibodies that recognize SMC or macrophages. In the carotid plaques, TUNEL stained a portion of cells with elongated nuclei (Figure 6, a and b), most of which contained muscle α-actin (Figure 6c), suggesting that they were apoptotic SMC. The α-actin$^+$/TUNEL$^+$ cells localized particularly in the fibrotic portion of the plaques, an area containing few lipid-laden foam cells but abundant connective tissue (Figure 6, a and b). Counterstaining with methyl green disclosed few nuclei in the fibrotic lesions (Figure 6, a and c). Interestingly, TUNEL clearly visualized some nuclei that were unstainable histochemically with methyl green (Figure 6b). In addition, more than 20% cells in the fibrotic area showed no immunoreactivity to the anti-α-actin antibody (Table 3) but some of them did exhibit TUNEL staining (Figure 6c), indicating that other mesenchymal cells or modulated, α-actin$^-$ SMC might undergo apoptosis as well. Approximately 22% of cells showed TUNEL$^+$ in this area (Table 3). The appearance of numerous TUNEL$^+$ cells in the fibrotic

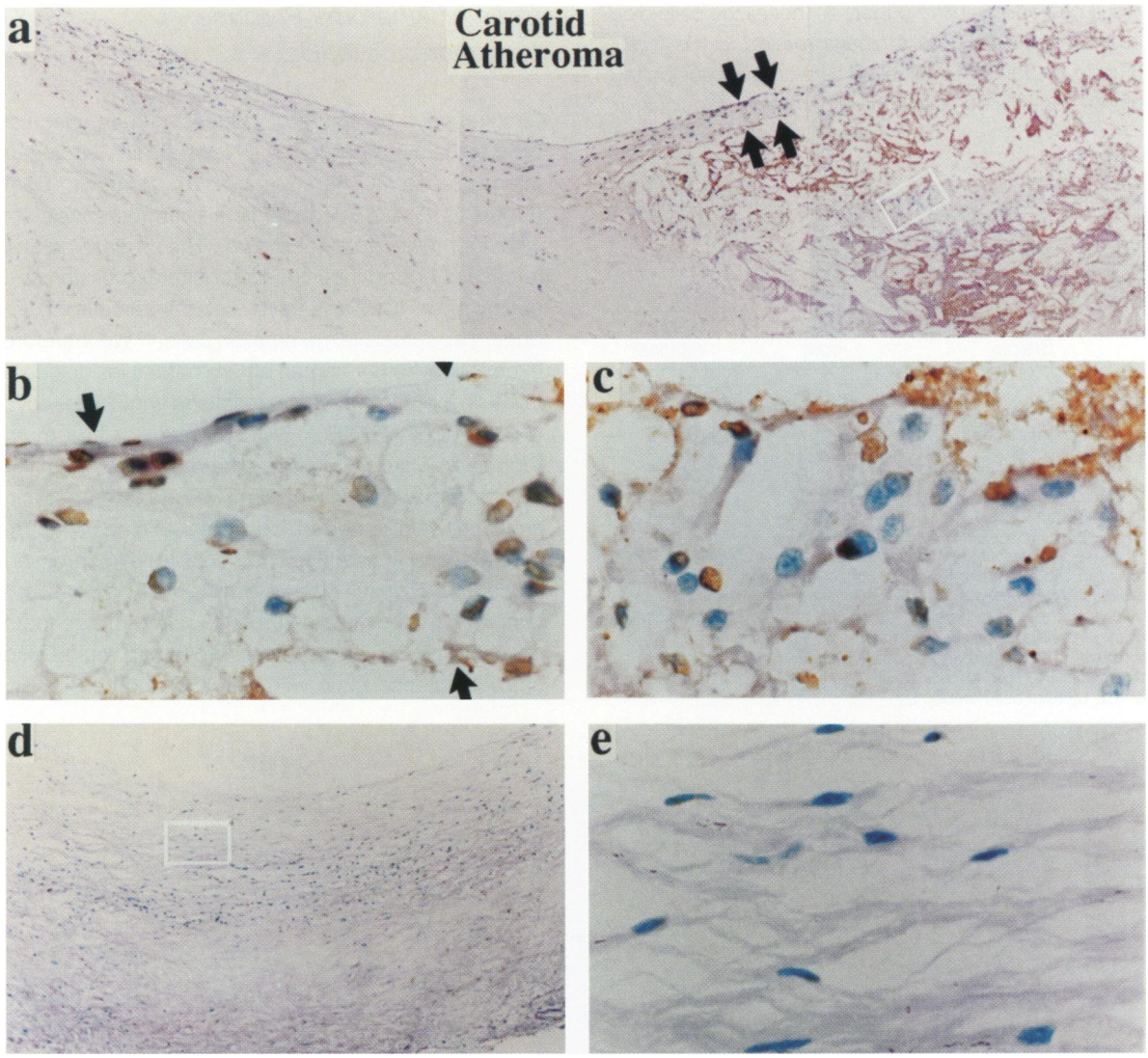

Figure 3. *TUNEL of atheromatous human carotid artery. Sections of human carotid atheroma were stained by TUNEL. Cells stained positively with TUNEL exhibited brown nuclei, and counterstaining with methyl green shows green-blue nuclei. Note that the thin fibrous cap (indicated by two pairs of arrows in* a) *contained many TUNEL$^+$ cells, which are shown at higher magnification in* b. *The arrows at* a *and* b *indicate the borders of the fibrous cap. The lipid core contained extracellular TUNEL-stainable DNA fragments (box in* a), *but few cells were stained with TUNEL in the adjacent normal tissue (*d *and* e). (b *and* c) *High power micrograph of the fibrous cap and lipid core regions indicated by arrows and box, respectively, in* a. (e) *High power micrograph of the boxed area in* d. *Original magnifications:* a *and* d, *×100;* b, c, *and* e, *×1000.*

lesions suggests that apoptosis may contribute to the formation of relatively hypocellular regions of fibrotic atherosclerotic lesions. Some α-actin$^+$ SMC also localized in the lipid-rich center and surrounding tissue where 10% cells stained α-actin$^+$/TUNEL$^+$ (Table 3).

In addition to SMC, macrophages derived from blood-borne monocytes also accumulate in atherosclerotic lesions.[4,6,10] Many of these plaque macrophages take up lipids and become lipid-laden foam cells characteristic of atherosclerosis. Double staining with TUNEL and anti-macrophage CD68 demonstrated numerous CD68$^+$/TUNEL$^+$ macrophages in plaques (Figure 7, Table 3), suggesting that macrophages undergo apoptosis in these lesions. In contrast to SMC, many TUNEL$^+$ macrophages clustered in the lipid core and had the appearance of lipid-laden foam cells (Figure 7). Only 4% of cells dispersed among SMC in the fibrotic area showed both immunoreactive CD68 and TUNEL stain (Table 3). Interestingly, in the macrophage clusters, some CD68$^+$/TUNEL$^+$ cells appeared to have released their intracellular contents including nucleic acids leading to formation of a small "necrotic center" containing both TUNEL$^+$ cells and extracellular TUNEL-detectable DNA fragments (Figures 3 and 7c). However, consistent with the

result presented above (Figure 3), not all the cells died, as CD68$^+$/TUNEL$^-$ cells clearly existed in this region (Figure 7c, Table 3), indicating a heterogeneous pattern of cell death occurring in the atheromatous lesions.

Table 3. *Expression of CD 68, Actin, and ICE in Apoptotic Cells Detected by TUNEL in Different Parts of Human Atheromatous Lesion*

Stains	% Positive cells	
	Lipid core	Fibrotic regions
TUNEL	43 ± 9	21 ± 5
CD68	60 ± 16	4 ± 6
TUNEL + CD68	38 ± 7	3 ± 6
α-Actin	33 ± 5	79 ± 8
TUNEL + actin	11 ± 6	22 ± 4
ICE	40 ± 14	34 ± 12
TUNEL + ICE	37 ± 4	23 ± 8

Serial sections of six human atheromas were stained by TUNEL followed by immunostaining with antibodies against macrophage CD68, SMC α-actin, and ICE. Cells were visualized with a two-color system to permit cell identification (see Materials and Methods). 400 cells were counted in random fields by high power microscopy. The percentage of positive stained cells was calculated by dividing number of cells positive to TUNEL, anti-CD68, α-actin, or ICE by total cell number. Data represent mean ± SD.

Cells of Atheroma Produce Immunoreactive ICE

Simultaneous exposure of cultured human vascular SMC to the cytokines IL-1β, IFN-γ and TNF-α induced internucleosomal fragmentation of genomic DNA (Figure 5 a) and morphological changes consistent with apoptosis (data not shown). The processing and release of endogenous IL-1β require expression and activation of ICE, an enzyme also implicated in inducing apoptosis in mammalian cells.[24] We therefore examined whether human atheromatous lesions contain ICE. Double staining colocalized immunoreactive ICE with both TUNEL$^+$ SMC (Figure 6d) and macrophages (Figure 7d) within atheroma. To confirm the results of double staining that was done on paraffin sections, we also performed single staining using frozen sections. Intimal lesions consistently expressed immunoreactive ICE, particularly in the lipid core containing numerous cholesterol-rich macrophage foam cells (Figure 8, a and b). The fibrous cap region also showed intense stain with anti-ICE (Figure 8b). By

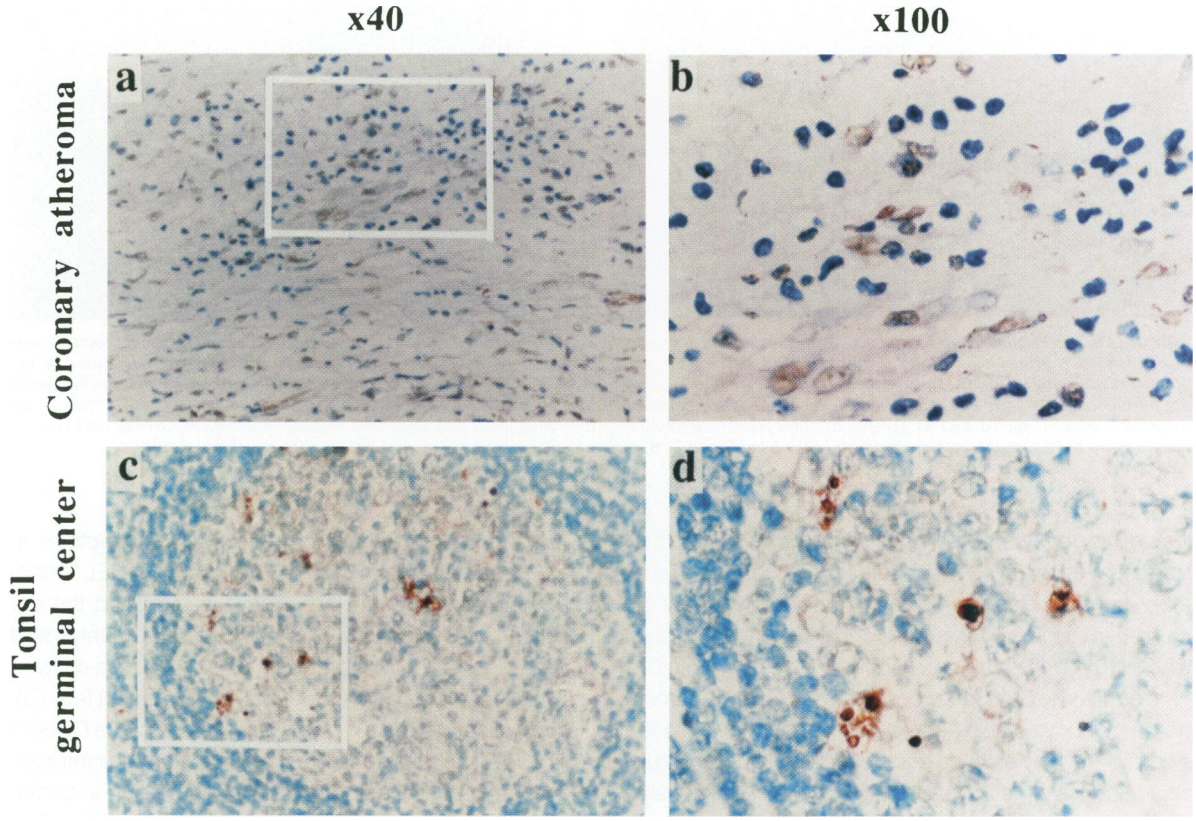

Figure 4. *Comparison of TUNEL staining in the intima of atheromatous coronary arteries and the germinal center of tonsil. Sections of human atheromatous coronary arteries* (a *and* b) *and normal tonsillar tissue* (c *and* d) *were stained with TUNEL. TUNEL$^+$ cells displayed brown, and TUNEL$^-$ cells green-blue nuclei. Note the presence of TUNEL$^+$ cells in atheroma* (a *and* b) *as well as the germinal center of tonsil lymphoid tissue* (c *and* d) *but not the tissue surrounding the germinal center* (c *and* d). b *and* d *show higher power micrographs of the regions indicated in* a *and* c. *Original magnifications:* a *and* c, ×100; b *and* d, ×1000.

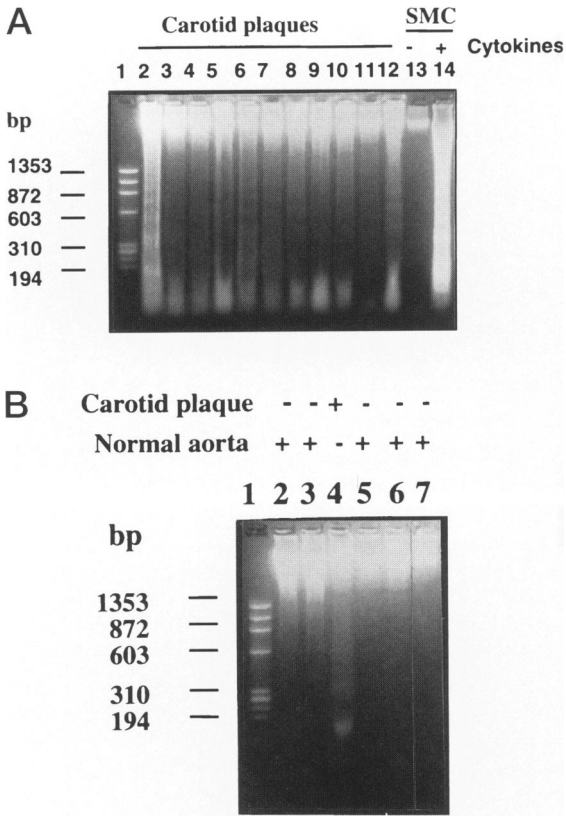

Figure 5. *Fragmentation of genomic DNA from human atheroma, normal aorta, and cultured SMC. Genomic DNA was isolated from carotid atheroma and non-atherosclerotic aortas or from cultured human aortic SMC treated with or without a combination of the cytokines, 100 U/ml IL-1β, 400 U/ml TNF-α, and 400 U/ml IFN-γ. DNA fragmentation was evaluated by electrophoresis in 1.8% agarose gel and visualized under UV light after staining with ethidium bromide.* (a) *Internucleosomal DNA fragmentation in the carotid atheroma and cytokine-stimulated SMC:* lane 1, *DNA size markers;* lanes 2–12, *atheromatous plaques from different patients;* lane 13, *SMC untreated;* lane 14, *SMC treated with the cytokines, IL-1β, TNF-α, and IFN-γ.* (b) *DNA integrity in normal aorta:* lane 1, *DNA size markers;* lanes 2, 3, 5–7, *normal aortic tissue from different individuals;* lane 4, *carotid atheroma for comparison.*

contrast, we found little immunoreactive ICE in non-atherosclerotic arterial tissue (Figure 8d). We noted that in the lesions containing both TUNEL$^+$ and TUNEL$^-$ cells, the anti-ICE antibody selectively reacted with the TUNEL$^+$ but not TUNEL$^-$ cells (Figure 9). In the cells with TUNEL$^+$/methyl green$^-$ nuclei, the anti-ICE stain appeared weak (Figure 9, b and c), suggesting that cells at the later, "mummified" stages of apoptosis contain only a low level of ICE.

In contrast to necrotic cells, cells undergoing apoptosis can maintain integrity of their plasma membrane, particularly at the earlier stages of the death, but phagocytes readily recognize and engulf these cells. We observed apparently intact TUNEL$^+$/ICE$^+$ cells in atherosclerotic lesions (Figure 9). Occasionally, we also found several TUNEL$^+$ nuclei in a single "giant" cell that reacted with the antibody against ICE (Figure 9d). This observation indicates the occurrence of phagocytosis of ICE$^+$/TUNEL$^+$ cells in the lesions.

Cells of Atheroma Express ICE mRNA

To confirm expression of the ICE gene in human atheroma, we analyzed RNA isolated from carotid atheromatous plaques by RT-PCR with a set of primers designed for ICE cDNA.[30] All four plaques tested, but not normal arteries, yielded a 399-bp product corresponding to ICE mRNA (Figure 10a). The PCR amplification appeared specific for ICE mRNA, as this procedure amplified no other species and produced no signal in the absence of input cDNA (Figure 10a). A similar analysis showed that both cultured human monocytes and SMC expressed ICE, although under the conditions used, the signal from monocytes appeared stronger than that from SMC (Figure 10b). For comparison, we also performed RT-PCR in the same RNA samples using another set of PCR primers for keratinocyte TG, an enzyme considered responsible for cross-linking of proteins during apoptosis.[35–37] After 35 cycles of PCR with this set of primers, we observed a cDNA product at the expected size for TG in SMC but not in monocytes (Figure 10b). These results suggest a difference in expression of death-regulating genes such as ICE and TG between monocytes/macrophages and SMC.

Discussion

This study sought evidence for apoptosis in human coronary and carotid atheroma. We observed that up to 30 to 40% of cells were TUNEL$^+$ in the markedly thickened intima of late-stage atheromatous arteries. Han et al[38] report similar results, which show that 10% cells in sclerotic lesions and 46% cells in the macrophage-rich area are TUNEL$^+$. Also, using an *in vitro* approach, Bennett and Schwartz[39] reported that a higher rate of apoptosis occurs in SMC derived from human atheroma compared with those from normal arteries. Taken together, these data imply that apoptosis may contribute to regulation of local cell accumulation in advanced human atheromatous lesions.

Such a high degree of TUNEL staining seems surprising, because the percentage of TUNEL$^+$ cells exceeds the rate (1 to 2%) of cell proliferation estimated in previous studies on the same types of lesions.[4,40] We have therefore considered the possibility that TUNEL overestimates the actual rate of apoptosis.

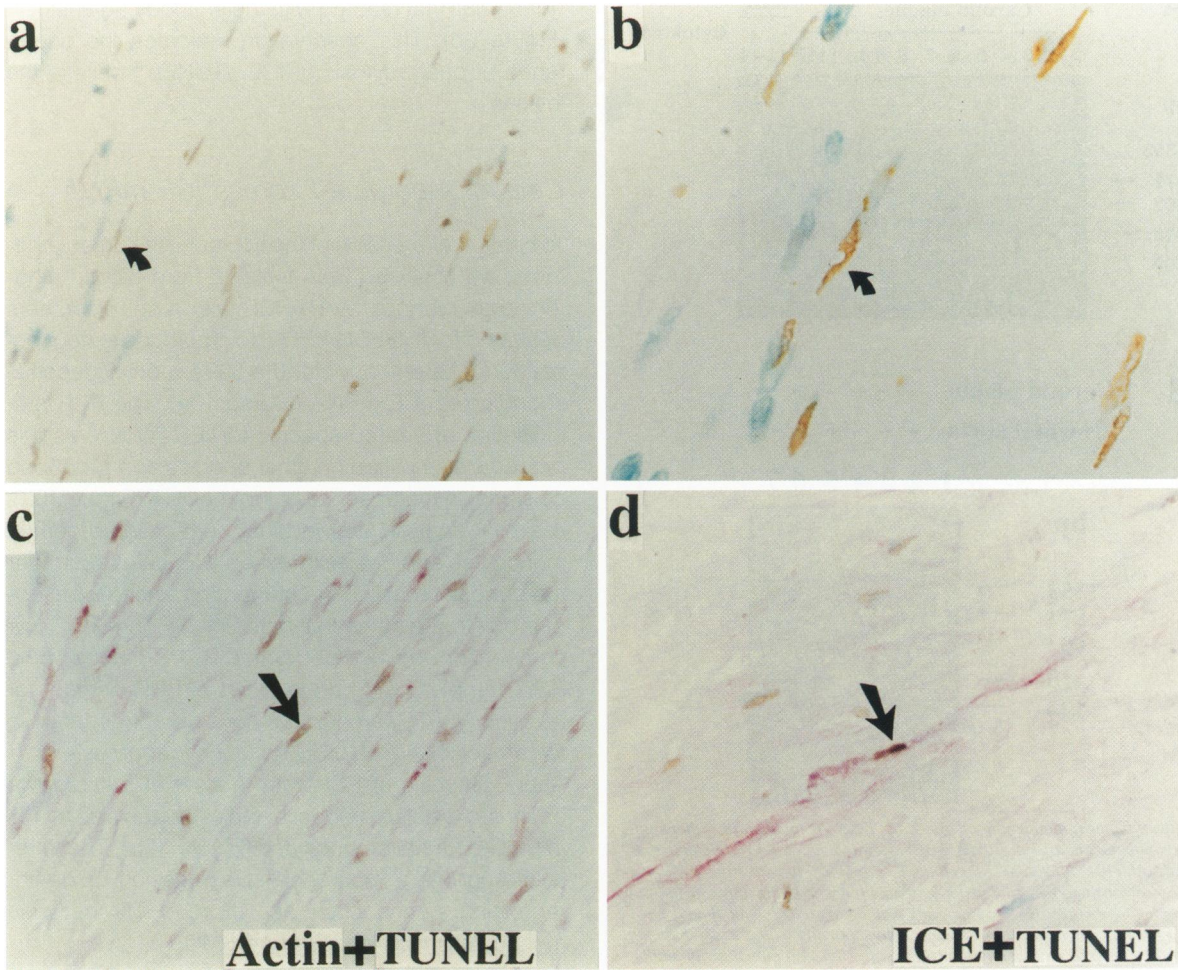

Figure 6. *Double staining with a combination of TUNEL and immunohistochemistry with anti-SMC actin and anti-ICE in fibrotic human carotid atheroma. Sections of human carotid plaques were stained with TUNEL* (a–d) *and then with anti-muscle actin* (c) *or anti-ICE* (d). *The TUNEL staining yielded brown nuclei as developed in the peroxidase-substrate system. The anti-actin immunostain was detected using anti-mouse IgG conjugated with biotin and an avidin-alkaline phosphatase-substrate system, yielding red cells. Counterstaining with methyl green yielded blue-green nuclei. Note that ICE colocalized with TUNEL$^+$ SMC in the fibrotic lesions* (c *and* d, larger arrows), *and some TUNEL$^+$ nuclei appeared pyknotic, binding little or no methyl green* (a *and* b, smaller arrows). (b) *High power micrograph of the area pointed out by an arrow in* a. *Original magnifications:* a, c, *and* d, *×400;* b, *×1000.*

However, the observations that 1) TUNEL stained nuclei of the cells with an apoptotic morphology and of those in atheromatous lesions but not of those in the normal arteries and 2) TUNEL staining depended on exogenous TdT argue against the possibility that the high percentage of TUNEL$^+$ cells results from nonspecific staining with TUNEL. The staining of cells in the germinal center but not in surrounding regions of tonsillar tissue also indicates the selectivity of the TUNEL technique used here. The data of morphological and genomic DNA analyses offer further support for the occurrence of apoptosis within plaques.

A high degree of apoptosis should cause a decrease in tissue cell mass.[20,41] We and others now confront an apparent paradox, that cells bearing markers of programmed death exceed those bearing markers of replication in a tissue classically characterized as hyperplastic. However, we must bear in mind that the persistence and sensitivity of the markers used for apoptosis and replication may vary. Moreover, as supported by data presented here, considerable dispersion in rates of cell replication and death may occur in space and in time during atherogenesis. Our data present a static view of advanced lesions at a given point in time, not an integrated picture over the life of the lesions. Rather than establishing absolute quantitative rates for apoptosis, we believe that these data should be used to support the concept that apoptosis occurs in advanced atheroma and should be added to our considerations of growth control and pathobiology of these lesions.

One possible contributor to the unexpectedly high frequency of cells, particularly SMC, bearing markers of apoptosis in atheroma may be prolonged persis-

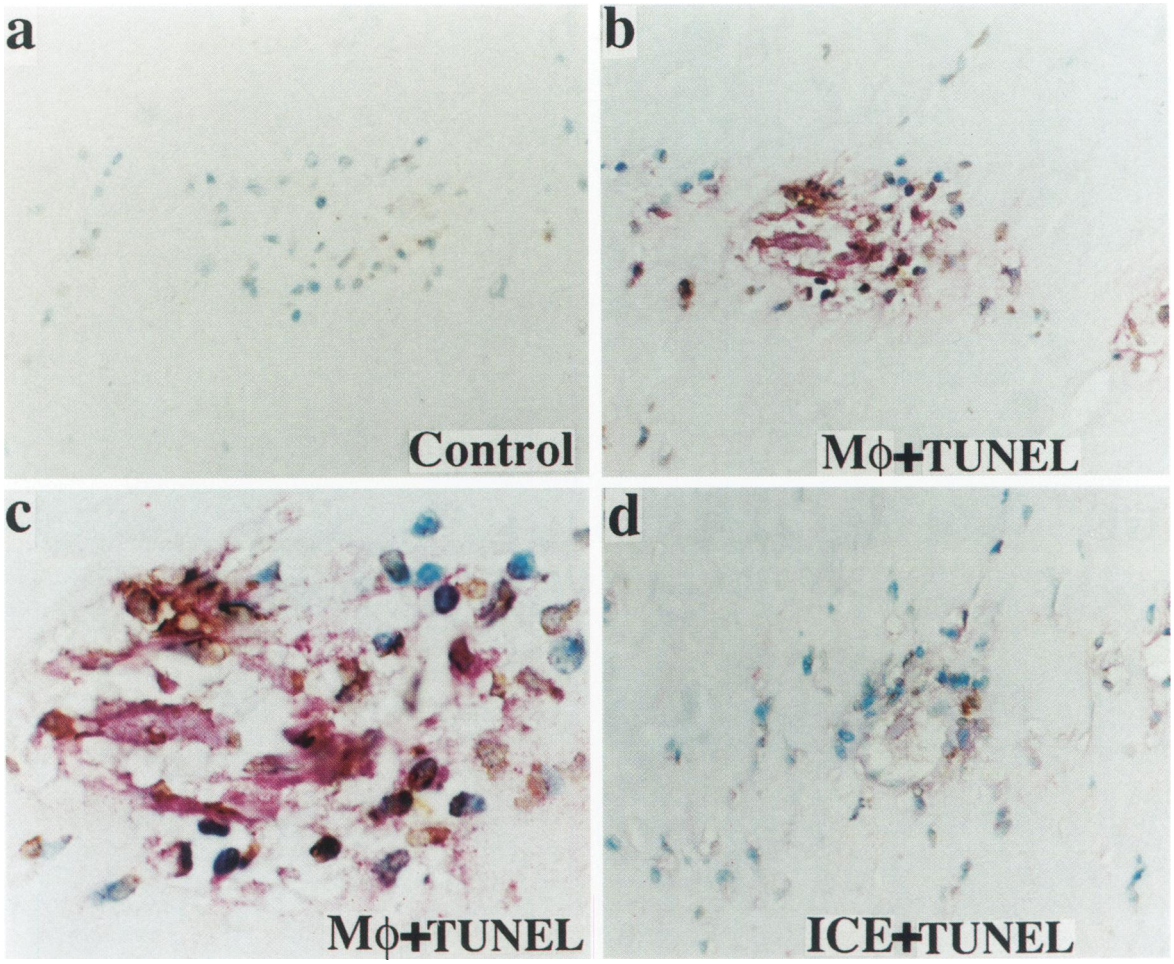

Figure 7. *Double staining with a combination of TUNEL and immunohistochemistry with anti-macrophage CD68 and anti-ICE in human carotid atheroma. Sections of human carotid plaques were stained with TUNEL* (**b–d**) *and thereafter with anti-CD68* (**b** *and* **c**) *or anti-ICE. The TUNEL stain yielded brown nuclei. Immunostain of anti-CD68 or ICE was visualized by using anti-mouse IgG conjugated with biotin and an avidin-alkaline phosphatase-substrate system, yielding red cells. Control staining was performed by using irrelevant IgG and omission of TdT in a serial section* (**a**). *Note CD68$^+$/TUNEL$^+$ macrophages clustered, forming a small "necrotic center"* (**b** *and* **c**) *and ICE colocalized with these cells* (**d**). **c** *High power micrograph of the macrophage cluster shown in* **b**. *Original magnifications:* **a**, **b**, *and* **d**, ×*400*; **c**, ×*1000*.

tence. Some apoptotic cells may not disappear from the atherosclerotic lesions, but accumulate in the fibrotic lesions in a "mummified" state. Phagocytes or adjacent cells can recognize and scavenge apoptotic cells, rendering them undetectable.[12,42,43] Visualization by TUNEL of cell nuclei not seen on routine histological staining indicates that some dying cells in atheroma may evade rapid phagocytosis and persist in the lesions. Protein cross-linking enzymes such as TG expressed constitutively by SMC[44] may stabilize these TUNEL$^+$ cells. This situation resembles that found in skin, where keratinocytes die via apoptosis without disappearance,[35,36] a precedent for the concept of "mummification" of apoptotic cells in atherosclerotic lesions as we propose here. In contrast with the case of SMC, some macrophages undergoing apoptosis may release their intracellular contents via cytolysis into the lipid-rich core of advanced atheroma. This process would account for the extracellular accumulation of TUNEL-stainable DNA fragments we observed in the lipid core of atheromatous lesions. Although we focus here on apoptosis as a novel mechanism of cell death in advanced atheroma, our data by no means exclude cell death by other means, which likely also occurs during atherogenesis, particularly in the lipid core.

Our observations further support the notion that apoptosis occurs in an inhomogeneous fashion in atheroma, leading to variation in the number of dying cells between different regions and cell populations in the arterial wall. Within atheroma both SMC[2] and macrophages[45,46] display considerable heterogeneity in phenotypes and biological properties. Various subpopulations of these cells may differ in their sensi-

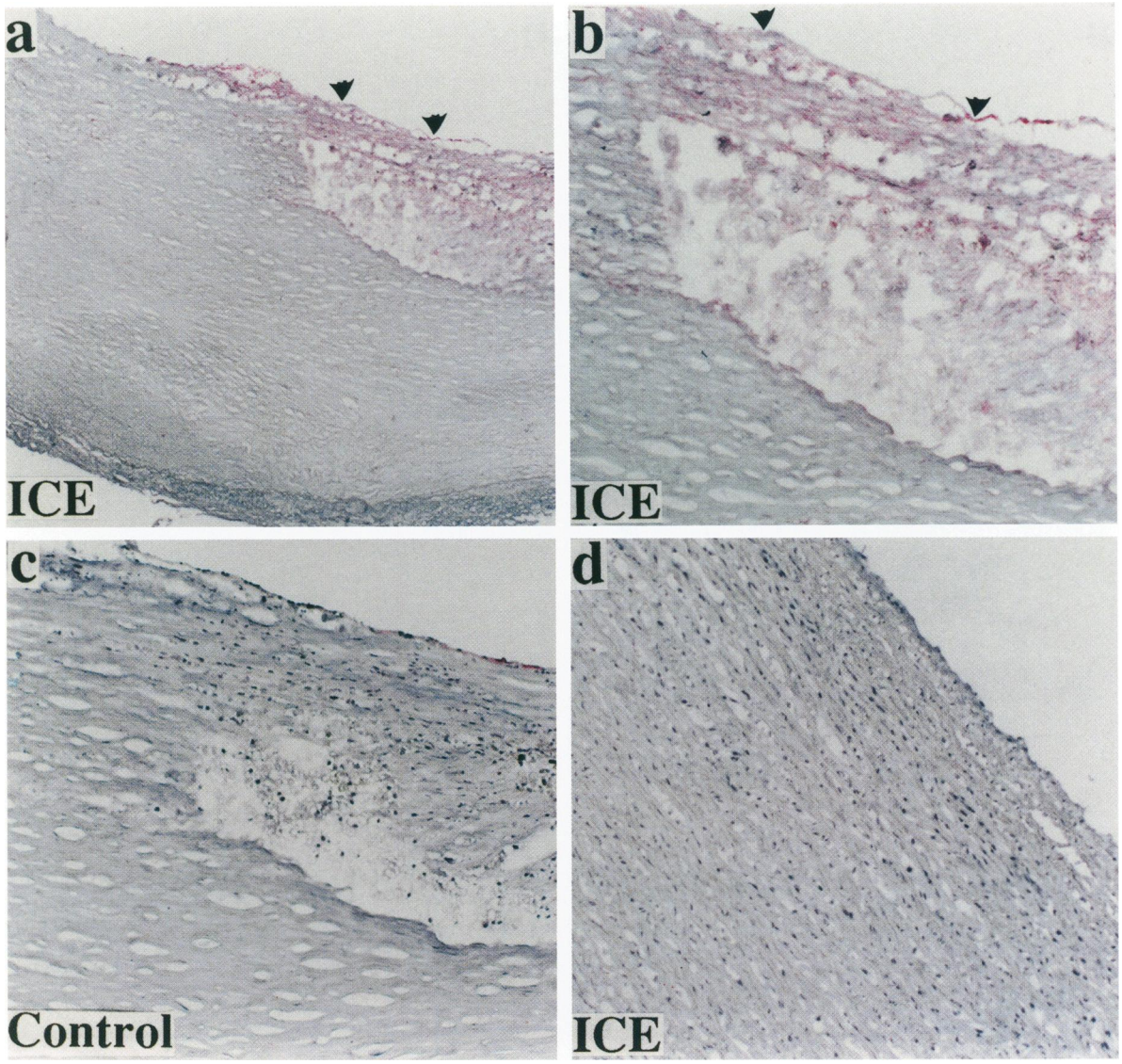

Figure 8. *Immunohistochemistry of ICE in human carotid atheroma and normal aorta. Frozen sections of the carotid atheromatous tissue* (a–c) *and normal aortic tissue* (d) *were stained with anti-ICE antibodies. The anti-ICE immunostain was detected using a biotin-conjugated second antibody anti-rabbit IgG and visualized by an avidin-alkaline phosphatase-substrate system, yielding red stain. Control staining was performed using non-immune rabbit serum* (c). *Note the intense anti-ICE stain localized in the lipid core and fibrous cap regions of atheroma* (a *and* b) *but not in the normal aorta* (d). (b) *High power micrograph of the region with the lipid core and fibrous cap shown in* a. *Original magnifications:* a, ×*100;* b–d, ×*400.*

tivities to or exposure to inducers of apoptosis. The finding that intimal SMC display a higher frequency of the TUNEL stain than do medial SMC points to a difference in apoptosis between the intimal and medial cells.

As alluded to above, the balance between cell proliferation and cell death may vary in time as well as space. In the earlier stages of lesion development and at certain critical times in the evolution of a mature lesion, cell proliferation may predominate. Cell death may become dominant in later stages of atherogenesis, as in the advanced lesions studied here, often obtained as surgical specimens because they were clinically active. Indeed, apoptotic cell death could directly influence aspects of arterial structure important for plaque stability. We now recognize that disruption of atherosclerotic plaques often cause the acute clinical manifestations. Areas of the fibrous cap of the lesion, prone to rupture, typically are thin and have few SMC.[47,48] SMC elaborate the constituents of the arterial extracellular matrix that determine the structural integrity of the fibrous cap of the plaque. Thus, apoptosis of SMC in these regions at later stages of plaque evolution may impede repair or maintenance of this matrix, and set the stage for plaque rupture.[47,48] Our current finding that consid-

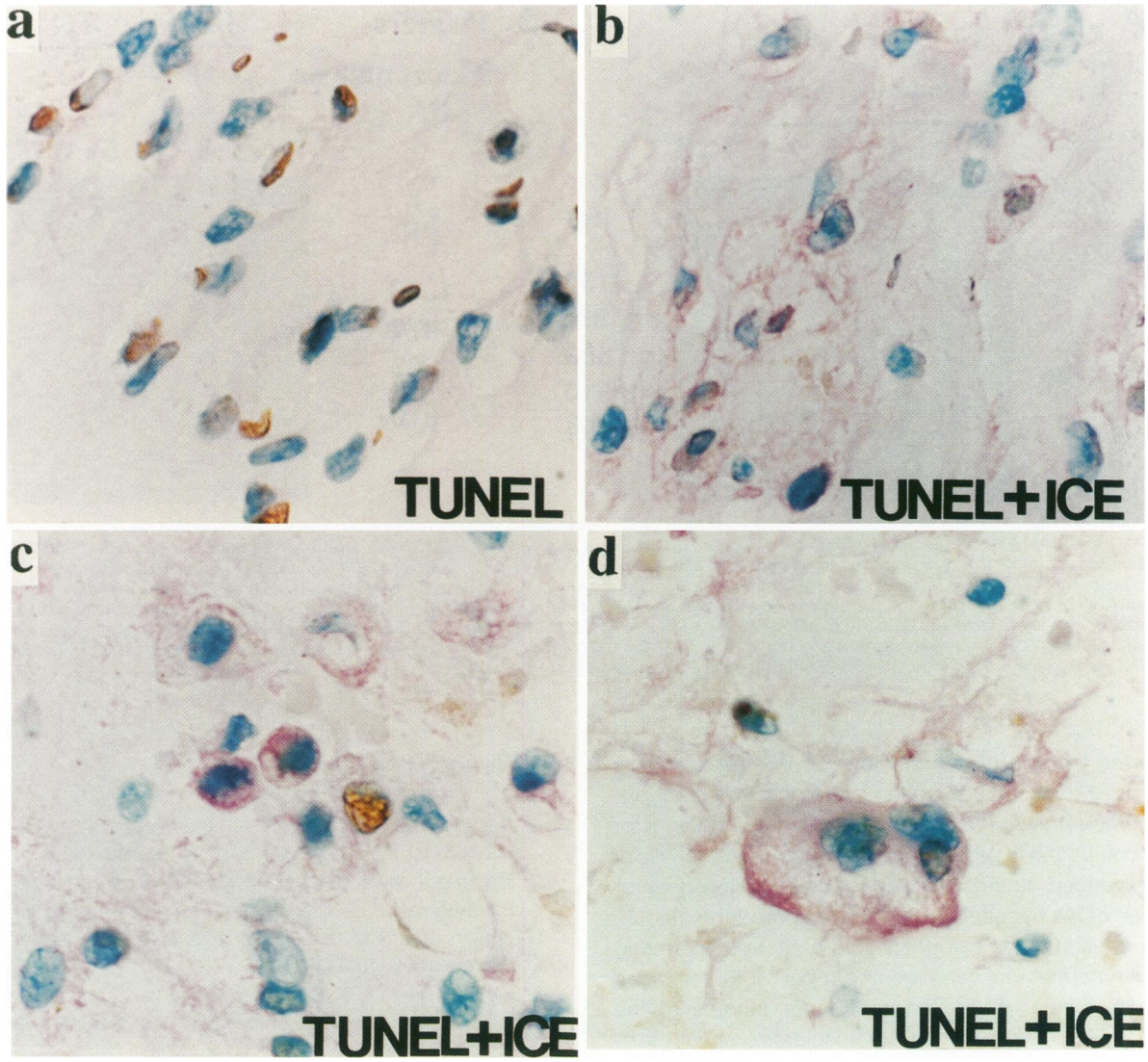

Figure 9. *Immunocytochemical characterization of ICE in TUNEL$^+$ cells of human carotid atheroma. Sections of carotid atheroma were stained with TUNEL alone or TUNEL$^+$ immunostaining. The TUNEL stain* (brown) *and immunostain* (red) *were developed in peroxidase- and alkaline phosphatase-substrate systems, respectively. Note the appearance of intact ICE$^+$/TUNEL$^+$ cells* (b, c, *and* d) *and an ICE$^+$ "giant" cell containing multiple TUNEL$^+$ nuclei* (d). *Original magnification:* $\times 1000$.

erable numbers of TUNEL$^+$ cells reside in the fibrous cap of human atheroma supports this hypothesis.

Many studies have defined signals that initiate and mechanisms that mediate apoptosis in other tissues, eg, induction of death-promoting genes or inhibition of death-suppressive genes.[13,20,49–51] The enzyme ICE, a mammalian homologue of the nematode *C. elegans* death gene, *ced*-3, induces fibroblast death after transfection with the ICE cDNA.[24] The present study colocalized ICE protein with macrophages and SMC bearing markers of apoptosis, suggesting that this or a related enzyme may play a similar role in the atherosclerotic lesions. An increase in IL-1β release occurs in macrophages undergoing apoptosis.[52] Our *in vitro* data show that IL-1β in combination with other proinflammatory cytokines such as TNF-α and IFN-γ induced apoptosis in cultured human SMC. The individual cytokines by themselves do not suffice to trigger apoptosis under these experimental conditions. Cells within plaques including macrophages and T cells can furnish TNF-α or IFN-γ.[53–55] However, demonstration of secretion of active IL-1β by SMC under usual conditions has proven difficult.[27] ICE may promote apoptosis by producing active IL-1β, which serves as an additional cytokine regulating this function of vascular cells.

In conclusion, these data highlight a role for programmed cell death in advanced human atheroma. Our results, together with those emerging from other laboratories, broaden the traditional view that empha-

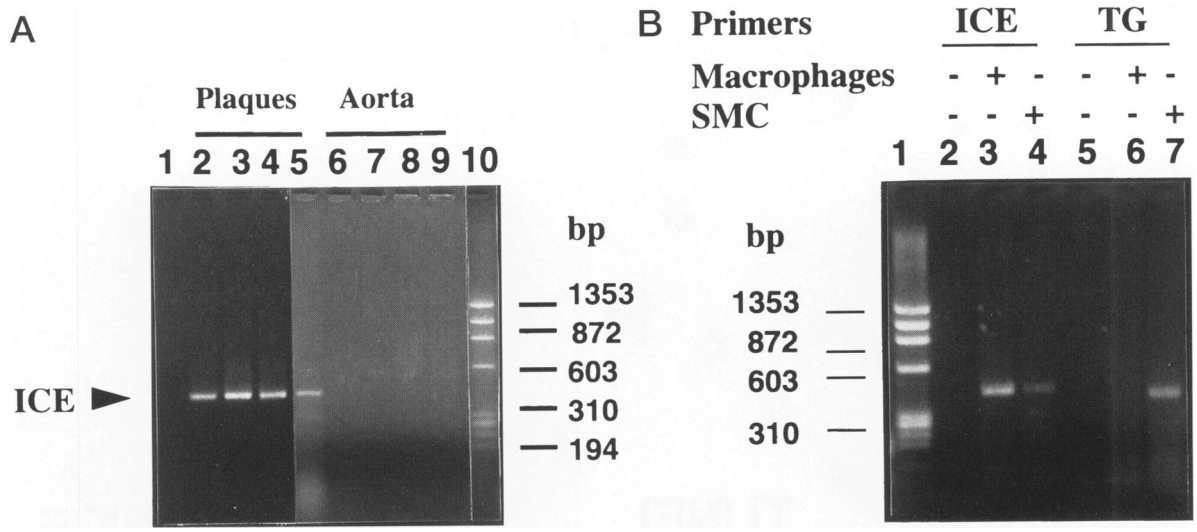

Figure 10. *Expression of ICE mRNA in human carotid atheroma, normal aorta, and in cultured human monocyte-derived macrophages and SMC. Total RNA was isolated from normal aorta and carotid atheroma and cultured cells. 200 ng RNA was reverse-transcribed into cDNA. The resulting ICE cDNA was then amplified by 35-cycle PCR with ICE primers (see Table 1), yielding a 399-bp band visible under UV light after electrophoresis of the PCR products in agarose gels containing ethidium bromide. A set of primers designed for TG (see Table 1) were also used in the parallel.* (a) *RT-PCR analysis of ICE in the carotid atheroma and normal aorta.* Lane 1, *without RNA;* lanes 2–5, *RNA from atheroma;* lanes 6–9, *from normal aorta;* lane 10, *DNA size markers.* (b) *RT-PCR analysis of ICE and TG in the cultured human monocyte-derived macrophages and SMC.* Lane 1, *DNA size markers;* lane 2, *without RNA for ICE;* lane 3, *RNA from macrophages for ICE;* lane 4, *from SMC for ICE;* lane 5, *without RNA for TG;* lane 6, *RNA from macrophages for TG;* lane 7, *from SMC for TG.*

sizes the role of cell proliferation in atherogenesis. These observations open up the possibility that apoptosis may act as an important, independent factor contributing to pathological changes characteristic of advanced atherosclerotic lesions including generation of the hypocellular fibrotic regions of the plaque, formation of the cytopenic lipid core, and interfering with normal maintenance and repair of the extracellular matrix of the lesion. Further studies of the pathobiology of human atherosclerosis should take apoptosis into account as a novel mechanism participating in plaque evolution and complication.

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